Interleukin-21 Augments the Efficacy of T-Cell Therapy by Eliciting Concurrent Cellular and Humoral Responses

Takekazu Iuchi,1,3 Seagal Teitz-Tennenbaum,1 Jianhua Huang,4 Bruce G. Redman,2 Steven D. Hughes,1 Mu Li,1 Guihua Jiang,1 Alfred E. Chang,1 and Qiao Li1

Departments of 1Surgery and 2Internal Medicine, University of Michigan Medical Center, Ann Arbor, Michigan; 3Shiga University of Medical Science, Shiga, Japan; 4PLA General Hospital, Beijing, People's Republic of China; and 5ZymoGenetics, Inc., Seattle, Washington

Abstract

Interleukin (IL)-21 modulates T-cell-associated, B-cell-associated, and natural killer cell-associated immunity. However, the potential of IL-21 to simultaneously stimulate cellular and humoral antitumor responses and the mechanisms involved have not yet been adequately explored. In this report, we examined the immune-modulating effect of IL-21 when used in vitro and its adjvant effects when administrated concomitantly with T-cell transfer for cancer therapy. Use of IL-21 in concert with IL-2 in culture up-regulated both type 1 and type 2 cytokine production of activated tumor-draining lymph node cells and enhanced their therapeutic efficacy. Administration of IL-21 and IL-2 as an adjuvant to T-cell transfer resulted in simultaneously elicited cellular and humoral responses. This concurrent response has led to effective regression of established pulmonary metastatic tumors and s.c. tumors. T-cell transfer plus IL-21/IL-2 administration conferred systemic immunity to the treated hosts. This was evident by the induction of protective immunity against tumor rechallenge, expansion of memory T cells, and significantly elevated serum levels of IFNγ and IL-10. Furthermore, we observed significantly enhanced tumor-associated antibody response after T-cell + IL-2 + IL-21 therapy. Cytotoxic antibody subclass IgG2b increased strikingly in the sera of treated animals; they bound specifically to MCA205 tumor cells, and such immune sera mediated tumor cell lysis in the presence of complement. Use of B-cell−deficient mice provided direct evidence that humoral responses contribute to T-cell + IL-2 + IL-21−elicited antitumor immunity. Collectively, these findings provide a rationale to evaluate the use of IL-21 in T-cell therapy of human cancers. [Cancer Res 2008;68(11):4431−41]

Introduction

Tumor-draining lymph node (TDLN) cells represent a source of lymphoid cells that can be secondarily sensitized by in vitro methods to generate effector cells capable of mediating regression of established tumors on adoptive transfer (1, 2). Clinical trials have been conducted in subjects with advanced cancers using adoptively transferred vaccine-primed lymph node cells (3–6). These trials have resulted in clinical responses in a limited percentage of patients. To enhance the efficacy of T-cell therapy, various strategies have been used as an adjunct to cell transfer. These combined therapies include cell transfer in concert with intra-tumoral expression of lymphotakin (7), tumor irradiation (8), dendritic cell vaccination (9), or blockade of certain coinhibitory molecules expressed in tumor cells, such as B7-H1 (10). The most useful adjuvant to T-cell transfer to date has been the exogenous administration of interleukin (IL)-2 (3, 4, 11, 12). However, the use of high doses of IL-2 has resulted in significant morbidity (3, 13) and can cause negative regulation of effector cells through activation-induced cell death (12, 14) or induce the proliferation of regulatory T cells (15).

IL-21 is a member of the IL-2 cytokine family. Reports on the immune modulation function of IL-21 have only begun to accumulate during the last few years. In recent studies, IL-21 was found to either induce cell-mediated tumor reactivity (16, 17), regulate B-cell differentiation and antibody responses (18–20), or modulate natural killer (NK) cell−associated immunity (21, 22), respectively. The pleiotropic effect of IL-21 on T and B cells makes it an interesting cytokine to examine in preclinical immunotherapy models.

Cellular and humoral responses represent two critical arms of immunity. We hypothesize that any successful anticancer treatment strategy will, in the final analysis, have to appropriately stimulate both humoral as well as cellular immunity. Currently, efforts to promote the effective induction of anticancer humoral responses of solid tumors have been significantly less than that to elicit cellular responses. Lack of induction of systemic and long-term antitumor immunity is one of the obstacles to the achievement of successful cancer immunotherapy. It is well accepted that antibody production and its persistence can promote the retention of long-term immunity (23–25). Therefore, any new reagent that can serve as an effective immune adjuvant to adoptively transferred T cells by boosting both cellular and humoral antitumor immunity represents a novel approach for human cancer treatment.

The prospect of IL-21 in simultaneously stimulating different immune cell subsets warrants further investigation. In this study, we examined the immune-modulating effect of IL-21 when used in vitro to generate effector T cells as well as its adjuvant effects when administrated in vivo with adoptive T-cell therapy.

Materials and Methods

Mice. Female C57BL/6 mice were from Harlan Laboratories or Charles River Laboratories. B-cell−deficient mice m/aMT Igh-6mIcgn on C57BL/6 background were from The Jackson Laboratory. All the animals were maintained in a pathogen-free environment and used at 8 wk of age or older. The University of Michigan Laboratory of Animal Medicine approved all the animal protocols.

Murine tumors. MCA205 murine tumor is 3-methylcholanthrene−induced fibrosarcoma that is syngeneic to C57BL/6 mice (26). EL-4 is a T-cell thymoma syngeneic to C57BL/6 mice. Pan-02, a pancreatic tumor
synergistic to C57BL/6 mice, was obtained from Dr. J. Norton (Stanford University, Palo Alto, CA). Tumor cells were prepared from solid tumors by enzymatic digestion (2).

**Tumor-draining lymph nodes.** MCA205 TDLNs were induced as previously described (2). CD4 and CD8 cells were purified from TDLN cells by using antibody-coupled Microbeads (Miltenyi Biotec). Purified cells were >90% CD4 or CD8 positive.

**TDLN cell activation and expansion.** TDLN cells at 2 × 10^6 in 10 mL complete medium (CM) per well were activated with anti-CD3 plus anti-CD28 monoclonal antibody (mAb; BD Biosciences) immobilized in six-well tissue culture plates (Costar) at 37°C with 5% CO2 for 2 d. After antibody activation, the cells were harvested and resuspended at 3 × 10^5/mL in CM containing human recombinant IL-2 (60 IU/mL; Chiron Therapeutics), IL-21 (1 μg/mL), or IL-2 plus IL-21 and incubated at 37°C with 5% CO2 for 3 d. Viable cell number was determined by trypan blue exclusion. Murine IL-21 was kindly provided by ZymoGenetics, Inc.

**Flow cytometry assay.** Cell surface molecule expression was analyzed by immunofluorescence assay using a FACSscan flow microfluorometer (Becton Dickson and Co.). The following antibodies were used: anti-CD3, anti-CD4, anti-CD8, anti-CD44, and anti-Iy-6G/Iy-6C (all from BD Biosciences). Matched isotype control mAbs were also used. Fluorescence-activated cell sorting (FACS) data analysis was performed using the CellQuest software (BD Biosciences). Binding of serum IgG2b to tumor cells was detected using FITC-anti-mouse IgG2b or isotype control (both from BD Biosciences) following the incubation of tumor cells with sera. Positive staining (MHC I expression) was done using FITC-anti-mouse H-2D^b (BD Biosciences).

**Cytokine release assay.** To measure cytokine release from cells, culture supernatants were collected at the end of cell expansion. To measure cytokines released in response to tumor stimulation, 1 × 10^6 activated and expanded TDLN cells were cocultured with 2.5 × 10^5 irradiated (6,000 cGy) MCA205 stimulator cells in 24-well tissue culture plates for 24 h at 37°C with 5% CO2. The culture supernatants were then collected. EL-4 tumor cells were used as specificity control. In some experiments, blood samples were collected for serum immunoglobulin quantitation kits (Bethyl Laboratories) as that used for total IgG detection.

**Adoptive T-cell therapy of pulmonary tumor.** C57BL/6 mice were inoculated by tail vein injection (i.v.) with 2 × 10^6 MCA205 tumor cells to establish pulmonary metastases. Three days after tumor inoculation, the tumor-bearing mice were treated with ex vivo activated and expanded TDLN cells via i.v. infusion. Commencing on the day of the cell transfer, IL-2 (40,000 IU) in 0.5 mL PBS twice daily and/or IL-21 (20 μg) in 0.5 mL PBS once daily were given ip for 4 d. Approximately 14 d after T-cell transfer, all mice were sacrificed, and lungs were harvested for enumeration of pulmonary metastatic nodules.

**Treatment of established s.c. tumor and tumor rechallenge.** C57BL/6 mice were inoculated s.c. in the mid-right flank with 1 × 10^6 viable MCA205 tumor cells in 0.1 mL HBSS. Three days after tumor inoculation, mice received 5 Gy of whole-body irradiation. Right after the irradiation, mice were infused iv. with activated and expanded TDLN cells followed by IL-2 (40,000 IU) in 0.5 mL PBS twice daily and/or IL-21 (20 μg) in 0.5 mL PBS once daily, were given ip for 4 d. Approximately 14 d after T-cell transfer, residual tumors were surgically removed from all the mice subjected to treatment. Tumor rechallenge was performed by injecting MCA205 tumor cells or EL-4 tumor cells. In some experiments, B-cell-deficient mice were used to test the role of antibody response in antitumor immunity.

**Antibody production assessment.** At the end of the treatment of established pulmonary metastases or s.c. tumor (day 17), mice from each group were sacrificed to collect blood samples for serum immunoglobulin detection. The concentration of total IgG and IgM in serum was measured by ELISA quantitation kits (Bethyl Laboratories). Serum IgG2a and IgG2b were also measured by ELISA. Tumor-specific IgG production was measured by indirect cellular ELISA. This procedure used the same ELISA quantitation kits (Bethyl Laboratories) as that used for total IgG detection with the following modifications. MCA205 tumor cells in CM at 0.25 × 10^6 cells per well were coated at 37°C overnight instead of goat anti-mouse IgG to capture IgG in the samples. To measure the relative level of tumor-specific IgG, the sample with the highest level of tumor-specific IgG was diluted in series so that a dilution-absorbance curve could be constructed, which would then be used as the standard curve in the indirect cellular ELISA.

**Immune sera and complement-mediated cytotoxicity.** Tumor cell lysis by immune sera and complement was performed by incubating the tumor cells with the sera in test tubes on ice for 1 h followed by cell culture in the presence of rabbit complement (Calbiochem) in a 37°C water bath for another hour. Viable cells were then counted under a microscope after trypan blue staining to calculate cell lysis. Alternatively, cytotoxicity was analyzed in 96-well flat-bottomed tissue culture plates (Corning, Inc.) and determined using the Quick Cell Proliferation Assay kit (BioVision) according to the manufacturer’s instructions. Absorbance values were measured via a multiwell spectrophotometer to evaluate cell lysis.

**Statistical analysis.** The significance of differences in numbers of metastatic nodules, the concentration of cytokines, and the concentration of immunoglobulin between experimental groups was determined using one-way ANOVA (Newman-Keuls post hoc test). Size of the tumor was analyzed at each time point by unpaired t test. All statistical analyses were performed using GraphPad Prism software. Statistically significant difference between two groups was achieved when P was <0.05.

**Results.**

**IL-21 in concert with IL-2 up-regulates both type 1 and type 2 cytokine production of activated TDLN cells and enhances their therapeutic efficacy.** We have previously shown that ~60% of the TDLN cells are CD3^+ T cells. Activation of these TDLN cells with anti-CD3 plus anti-CD28 combination followed by IL-2 expansion results in therapeutic effector cells (>90% CD3^+; refs. 27–29). The therapeutic efficacy of these effector cells on adoptive transfer is related to the amount of type 1 cytokines (e.g., IFNγ) and granulocyte macrophage colony-stimulating factor secreted by these cells in response to tumor antigen stimulation.

Cytokine profiles released by TDLN cells cultured in IL-2 and/or IL-21 following anti-CD3/anti-CD28 activation were analyzed. In the culture supernatants, as shown in Fig. 1A, although IL-21 alone showed similar effects as IL-2 in stimulating IFNγ and IL-10 production by unfractionated TDLN cells or purified CD4^+ or CD8^+ TDLN cells, IL-21 significantly increased IFNγ and IL-10 production synergistically with IL-2 in all three TDLN cell populations. Using intracellular staining, we found that after culture in IL-2, IL-21, or IL-2 plus IL-21, the IFNγ-producing CD4 cells were 16%, 23%, and 27%, respectively, showing an enhanced IFNγ production induced by the use of IL-21 (Fig. 1B). This phenomenon was more evident in cultured CD8 cells. The IFNγ-producing CD8 cells were increased from 4% (IL-2 alone) to 12% (IL-21 alone) and then to 20% when IL-21 was used in combination with IL-2 (Fig. 1B). In parallel, intracellular staining also confirmed IL-21–augmented IL-10 production (Fig. 1C). This was also found more evident in cultured CD8 cells. We then measured the cytokines released by cultured TDLN cells in response to tumor antigen. As shown in Fig. 1D, TDLN cells cultured with IL-21, either alone or combined with IL-2, significantly elevated IFNγ secretion in response to specific tumor stimulation (P < 0.01). In the same experiments, IL-21 synergistically increased IL-10 production with IL-2. Taken together, these data show that IL-21, when used in concert with IL-2, can modulate both type 1 (e.g., IFNγ) and type 2 (e.g., IL-10) cytokine production by TDLN cells.

We proceed by checking the therapeutic efficacy of IL-2– and/or IL-21–cultured TDLN cells. In vivo antitumor reactivity...
Figure 1. IL-21 in synergy with IL-2 modulates both type 1 and type 2 cytokine secretion of TDLN cells. A, TDLN cells were activated with anti-CD3 and anti-CD28 for 2 d followed by culture in IL-2 (60 IU/mL), IL-21 (1 μg/mL), or IL-2 plus IL-21 for 3 d. Unfractionated TDLN cells and purified CD4 or CD8 TDLN cells were used in these assays. Culture supernatants were collected at the end of cell expansion to detect the released cytokines by ELISA. *, P < 0.05, comparing IL-2 plus IL-21 cultured cells with any other groups in the same chart. Antibody-activated and IL-2/IL-21–cultured TDLN cells were stained for intracellular IFNγ (B) and IL-10 (C) and analyzed by FACS. For intracellular staining of cytokines, phorbol 12-myristate 13-acetate (10 ng/mL), ionomycin (500 ng/mL), and GolgiStop (1 μL/mL) or GolgiPlug (1 μL/mL) were added to cell culture in CM and incubated for 4 to 5 h before staining. D, cytokine secretion of IL-2/IL-21–cultured TDLN cells in response to tumor stimulation. Antibody-activated and IL-2 and/or IL-21–cultured TDLN cells were cocultured with irradiated autologous MCA205 tumor cells or EL-4 tumor cells (specificity control). Twenty-four hours later, the supernatants were collected and analyzed for cytokine production. *, P < 0.01, compared with IL-2–cultured TDLN cells; **, P < 0.001, compared with any other groups in IL-10 secretion. Data are representative of two independent experiments.
of antibody-activated TDLN cells cultured in IL-2 and/or IL-21 was examined in an adoptive immunotherapy model. As illustrated in Fig. 2, TDLN cells cultured in IL-2 or IL-21, respectively, mediated tumor regression to a similar extent. However, the number of pulmonary metastases was significantly reduced after the transfer of TDLN cells cultured in IL-2 plus IL-21 compared with an equal number of TDLN cells cultured with each cytokine alone (P < 0.05). These experiments showed that IL-21 in concert with IL-2 can markedly enhance the therapeutic efficacy of the TDLN cells.

**Adjuvant effect of IL-21 administration in adoptive T-cell therapy.** We evaluated the in vivo effects of IL-21 administration with adoptive T-cell transfer in mediating the regression of established tumors. Tumor-bearing mice were treated with adoptive transfer of antibody-activated and IL-2–cultured TDLN cells with IL-21 administration. The initial experiments were performed without exogenous IL-2 administration (Fig. 3A). Various doses of T cells were transferred with administration of different concentrations of IL-21. When equal numbers (e.g., 1 × 10⁶) of T cells were transferred, increasing the concentration of IL-21 administered from 20 to 40 μg/d significantly increased the therapeutic efficacy by reducing the number of pulmonary metastases. On the other hand, when IL-21 was administered at 20 μg/d, increasing the transferred T-cell number from 1 × 10⁶ to 3 × 10⁶ likewise significantly increased the therapeutic efficacy. These experiments are thus supportive of the conclusion that adoptively transferred TDLN cells and administrated IL-21 additively contribute to the antitumor efficacy mediated by the combined therapy. When a certain concentration of IL-21 was administrated as an adjuvant, improved therapeutic efficacy can be achieved by increasing the T-cell doses transferred.

We then compared the adjuvant effect of IL-21 with IL-2. We also used IL-21 plus IL-2 to examine the potential of this cytokine combination to further augment the antitumor efficacy of adoptively transferred T cells. This would underscore the requirements for IL-21 administration or IL-21/IL-2 coadministration as an effective adjuvant to T-cell therapy. In Fig. 3B, IL-2 or IL-21 (20 μg/d) administration alone revealed no therapeutic effects. When 3 × 10⁶ of T cells were transferred, T-cell plus IL-21 administration showed superior antitumor activity to T-cell plus IL-2 administration, and this improved antitumor response was further enhanced by using IL-2 plus IL-21. When a suboptimal dose of T cells was transferred (1 × 10⁶; Fig. 3C), IL-21 administration significantly improved the therapeutic efficacy of adoptively transferred TDLN cells in conjunction with IL-2 (P < 0.05). These experiments show that IL-21 can be used alone or in combination with IL-2 as an effective adjuvant in adoptive cellular therapy.

To confirm the adjuvant effect of IL-21 and its cooperation with IL-2, the therapeutic efficacy of adoptively transferred TDLN cells plus IL-2 and/or IL-21 administration was evaluated in s.c. tumor models in the following experiments. Figure 3D shows that using IL-21 as adjuvant in T-cell transfer inhibited tumor growth to a greater extent than using IL-2, and the use of IL-2 plus IL-21 further enhanced the suppression of tumor growth (P < 0.05).

**T-cell plus IL-21 and IL-2 therapy confers systemic immunity to the treated hosts.** In both the pulmonary metastasis and the s.c. tumor models described above, the combined therapy with T-cell transfer plus IL-21 and IL-2 administration has been found to be more potent in mediating established tumor regression than T-cell plus IL-2 or IL-21 therapy. We then performed a series of experiments to test our hypothesis that systemic immunity is conferred by the cytokine administration during T-cell therapy.

We examined the protective antitumor immunity, which may be established by the T-cell plus IL-21 and IL-2 therapy. As shown in Fig. 4A, treatment with T-cell transfer plus IL-2 and/or IL-21 induced broad host immunity because all the mice subjected to treatment showed certain protective immunity against the irrelevant EL-4 tumor cell challenge as well as the MCA205 tumor rechallenge compared with the naive mice. However, IL-21 treatment significantly enhanced the protective immunity against the MCA205 tumor. On day 24 (7 days after tumor rechallenge), MCA205 tumor growth was significantly slower (P < 0.05) in mice treated with T cells plus IL-21 compared with mice treated with T cells plus IL-2, whereas tumor barely grew in animals subjected to T-cell plus IL-2 and IL-21 therapy. At a later time point on day 32 (15 days after tumor rechallenge), whereas MCA205 tumor continued growing aggressively in the control mice that had received no previous treatment, its growth remained significantly retarded in all the animals subjected to T-cell plus IL-2 or IL-21 therapy. Notable were the mice subjected to T-cell plus IL-21 and IL-2 therapy that remained tumor-free, showing a long-term protective immunity conferred by the combined therapy.

To investigate the protective nature of the immune response observed after adoptive T-cell transfer plus IL-2 and IL-21 administration, we analyzed the percentage of memory T cells in peripheral blood mononuclear cells (PBMC) isolated from s.c. tumor-bearing animals subjected to T-cell therapy accompanied by IL-2 and IL-21 injection. As illustrated in Fig. 4B, compared with the nontreated control groups, the percentages of CD3^+CD4^+ and CD3^+Ly-6G/Ly-6C^+ memory T cells within the CD3^+ T-cell population were dramatically increased from 23% to 61% and from 14% to 27%, respectively.

In these experiments, we also assayed the levels of IFNγ and IL-10 in the serum at the end of therapy. As revealed in Fig. 4C,
both IFNγ and IL-10 levels were significantly higher in the serum of the animals subjected to T-cell + IL-21 or T-cell + IL-21 + IL-2 treatments compared with animals that had received T-cell + IL-2 therapy.

Collectively, these experiments indicate that T-cell + IL-21 + IL-2 therapy confers superior systemic immunity to treated animals. This conclusion is evident by the induction of protective antitumor immunity against tumor rechallenge, increased numbers of memory T cells, and significantly elevated serum levels of both IFNγ and IL-10 generated by adoptive T-cell transfer + IL-21 + IL-2 administration in tumor-bearing hosts.

Antibody and B cells contribute significantly to T cell/IL-2/IL-21–elicited antitumor immunity. In addition to T cells, antibodies and B cells may play important roles in T-cell + IL-2 + IL-21–induced antitumor activity. We therefore evaluated humoral responses induced by administrated IL-21 and/or IL-2 as adjuvants in T-cell therapy. Blood samples were collected at the end of T-cell plus IL-2 and/or IL-21 therapy either from pulmonary metastasis-bearing mice (Fig. 5A) or from s.c. tumor-bearing mice (Fig. 5B) and assessed for serum total IgG and IgM as well as tumor-specific IgG production. In both tumor models, total serum IgG and IgM concentrations in the hosts treated with transferred T cells plus

![Graph depicting the antitumor efficacy of adoptively transferred TDLN cells.](image)

**Figure 3.** IL-21 administration augments the antitumor efficacy of adoptively transferred TDLN cells in the pulmonary metastatic and s.c. tumor models. MCA205 TDLN cells were activated with anti-CD3/anti-CD28 followed by expansion in IL-2. Mice with 3-d established MCA205 pulmonary metastases were treated by adoptive transfer of these effector TDLN cells at the indicated doses accomplished by IL-21 administration. IL-21 was administrated i.p. at the indicated doses for 4 d starting on the day when adoptive T-cell transfer was performed without (A) or with (B and C) IL-2 administration. Lungs were harvested and the pulmonary metastases were enumerated 15 d after tumor inoculation. D, IL-21 administration during TDLN cell transfer suppresses the growth of s.c. tumor in synergy with IL-2. Three days after MCA205 tumor injection s.c. on the right flank, mice were exposed to whole-body irradiation at 5 Gy. Immediately after irradiation, antibody-activated and IL-2–cultured MCA205 TDLN cells were transferred into the tumor-bearing mice with IL-2 (4 × 10^4 IU twice daily) and/or IL-21 (20 µg daily) i.p. administration for 4 d. P < 0.05 on day 17 comparing IL-2 plus IL-21 with IL-2 or IL-21 alone. Data are representative of two independently performed experiments.
administrated IL-2 and IL-21 were found significantly higher than any other groups. In the s.c. tumor model (Fig. 5B), T-cell plus IL-21 treatment induced significantly more IgG and IgM production than T-cell plus IL-2 administration. Additionally, assessment of tumor-specific IgG in serum by using MCA205 tumor cells in place of anti-IgG as a capture reagent in ELISA has shown (the last column of Fig. 5A and B) that, in both tumor models, mice that had received T-cell + IL-2 + IL-21 treatment produced significantly higher levels of tumor-specific IgG than mice treated with T cells plus IL-2 or IL-21. These experiments indicate that IL-21 in combination with IL-2 could serve as an excellent adjuvant to stimulate effective humoral responses in tumor-bearing hosts subjected to T-cell plus the cytokine combination therapy.

The enhanced antibody response may represent an additional mechanism involved in the therapeutic efficacy of TDLN cells with IL-21. To provide direct evidence that humoral responses truly contribute to T-cell + IL-2 + IL-21–elicited antitumor immunity, we proceeded to perform therapy experiments in B-cell–deficient mice. In these experiments, the adoptively transferred T cells were wild-type (wt) TDLN cells activated with anti-CD3 plus anti-CD28 followed by IL-2 expansion. They were >90% CD3+ T cells (27–29). The therapeutic efficacy of T-cell + IL-2 + IL-21 combined therapy in the B−/− mice was evaluated and compared with wt mice. Figure 5C shows that although T-cell + IL-2 + IL-21 therapy drastically inhibited tumor growth both in the wt and in the B−/− hosts (P < 0.0001, compared with the respective nontreated controls), there was a significant difference in the therapeutic efficacy between the two hosts subjected to the identical T-cell + IL-2 + IL-21 therapy. Tumor growth inhibition was significantly diminished in the B−/− mice compared with wt mice (P = 0.0071) after the combined treatment. These experiments show the supportive role of B cells in T-cell + IL-2 + IL-21–elicited antitumor immunity.
To define the possible mechanisms underlying the antitumor effects of antibodies, we tested the presence of cytotoxic antibody subclasses (e.g., IgG2a and IgG2b) in the serum of MCA205 tumor-bearing animals subjected to MCA205 TDLN T-cell and IL-2 + IL-21 therapy in Fig. 5C. Sera from B−/− mice served as controls. Whereas IgG2a was not detectable within the range of the test used (data not shown), sera collected from the T-cell + IL-2 + IL-21–treated wt mice showed markedly increased levels of IgG2b compared with the sera from nontreated animals (Fig. 5D).

We then tested the binding of IgG2b to tumor cells by flow cytometry. As revealed in Fig. 6A, serum IgG2b from each of the four wt mice subjected to T-cell + IL-2 + IL-21 therapy showed specific binding to MCA205 tumor cells (second panel).

A recent report (30) showed that IgG2b can mediate hyperacute rejection in rat cardiac xenografts in a complement-dependent manner. We proceeded by examining if the immune sera collected in Fig. 5C can mediate MCA205 tumor cell lysis in the presence of complement. These sera contained significantly increased amounts

Figure 5. Humoral responses contribute significantly to T-cell + IL-2 + IL-21–elicited antitumor immunity. A and B, significantly stimulated antibody production by IL-21 and IL-2 administration during T-cell therapy. Blood samples were collected at the end of T-cell plus IL-2 and/or IL-21 therapy either from pulmonary metastasis-bearing mice (A) or from s.c. tumor-bearing mice (B) and assessed for serum total IgG and IgM as well as tumor-specific IgG production using ELISA as described in Materials and Methods. C, requirement for B cells in T-cell + IL-2 + IL-21–elicited antitumor immunity. S.c. tumor models were established in wt mice or in B−/− mice and then treated with T-cell adoptive transfer plus IL-2 and IL-21 administration as in Fig. 3D. Similar data were generated in a second experiment.
of IgG2b (Fig. 6A), which bind specifically to MCA205 cells (Fig. 6B). As indicated in Fig. 6C, whereas sera from two nontreated control mice plus complement showed no MCA205 cell lysis compared with the use of complement alone (P > 0.05), sera from three mice subjected to T-cell + IL-2 + IL-21 therapy all mediated significant (P < 0.01) MCA205 tumor cell killing in the presence of complement. Use of Pan-02 tumor cells confirmed that this tumor killing was immunologically specific. Alternatively, antibody-mediated cytotoxicity was analyzed in 96-well culture plates using a different assay. In this assay, a special substrate, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1), was added to tumor cell cultures at the end of immune sera and complement incubation. The assay is based on the cleavage of WST-1 to formazan dye by cellular mitochondrial dehydrogenases. Because cleavage of WST-1 to formazan dye occurs only in viable cells, the amount of dye produced (in absorbance values) directly correlates with the number of viable cells present in the culture. As shown in Fig. 6D, immune sera from all of the six mice subjected to T-cell + IL-2 + IL-21 therapy each effectively lysed MCA205 tumor cells. This was indicated by the significantly (P < 0.01) reduced absorbance values in these groups. These experiments verified the immune sera plus complement-mediated tumor cell lysis data measured by direct viable cell counting under the microscope (Fig. 6C).

**Discussion**

Administration of IL-21 as monotherapy was found to be effective in attaining durable tumor immunity (16). However, potent antitumor activity by administration of proinflammatory cytokines as monotherapy is often achieved at the expense of unacceptable toxicity (31). Combined therapy involving IL-21 has been successful in several studies. For example, it was shown that induction of tumor cell apoptosis by agonistic mAb against DR5 combined with IL-21 treatment suppressed tumor growth (32). In addition, IL-21 in a synergistic manner with IL-15 augmented the DNA vaccine-induced immune responses to HIV compared with those elicited by immunization in the presence of either cytokine alone (33). IL-21 and IL-15 were also found to synergistically regulate CD8+ T-cell expansion and function (34). A study performed by He and colleagues (35) examined the use of IL-21 and IL-2 in combination with a tumor antigen vaccine and lymphopenic conditioning in cancer therapy. Their study showed a significant cellular response induced by the combined therapy; however, no humoral responses were described. Another report showing the induction of humoral antitumor responses with IL-21 therapy involved administration of IL-21 plasmid i.v. into mice bearing tumors (36). That report did not involve adoptive T-cell transfer. Recently, Daga and colleagues (37) assessed the ability of IL-21–transduced glioma cells to trigger intracranial tumor rejection.

*Figure 6.* IgG2b increases in the sera of T-cell + IL-2 + IL-21–treated animals and binds specifically to MCA205 tumor cells, and such immune sera mediate tumor cell lysis in the presence of complement. A, obviously increased IgG2b in the sera of animals subjected to T-cell + IL-2 + IL-21 therapy. Sera collected from the animals at the end of therapy in Fig. 5C were tested for IgG2b using ELISA. Two mice from each experiment group were examined in each test. Two tests were performed. Therefore, data of the two T-cell + IL-2 + IL-21–treated wt mice are representative of four mice tested in this group of total five to six mice. B, serum IgG2b from immune mice specifically binds to MCA205 tumor cells. MCA205 tumor cells were incubated with the sera from each of the four mice (indicated by 1, 2, 3, and 4) subjected to T-cell + IL-2 + IL-21 therapy in Fig. 5C. Bound serum IgG2b onto the tumor cells was then detected by secondary antibody (Ab) FITC-anti-mouse IgG2b. FITC-coupled secondary antibody isotype-matching control was also used. Use of secondary antibody alone without preincubation with the sera revealed no nonspecific binding to tumor cells. Direct staining using FITC-anti-mouse MHC I served as positive control in the system. Furthermore, an irrelevant tumor cell line Pan-02 was used to verify that binding of IgG2b from immune sera to MCA205 cells was tumor specific. Number within each immunofluorescence histogram indicates the percentage of viable cells stained positive with the FITC-coupled antibodies.
Nearly 100% of the animals receiving a primary implant of IL-21–transduced cells rejected the implant, and 76% of these animals survived to a subsequent rechallenge with parental glioma cells. Antibody responses were found crucial for glioma immunotherapy by IL-21–secreting cells, as immunotherapy was ineffective in syngeneic B-cell–deficient mice. That study did not involve effector T-cell infusion or IL-2 administration. We report in this study that the use of IL-21 in concert with IL-2 as an adjuvant to T-cell therapy has resulted in concurrent cellular and humoral responses. This response is associated with the up-regulation of both type 1 (IFN-γ) and type 2 (IL-10) cytokines and has led to effective therapeutic efficacy and long-term systemic immunity against cancer.

We reported that the in vitro cytokine profiles released by effector T cells when cocultured with tumor cells are predictive of their ability to mediate tumor regression in vivo (1–3, 27, 38). In this study, use of IL-21 in addition to IL-2 augmented both IFNγ and IL-10 production. The role of IL-10 in the antitumor response mediated by the transferred cells needs to be further explored. IL-10 is an important immunoregulatory cytokine that plays a critical role in initiating and maintaining antibody responses (39–41). The enhanced IL-10 production may be correlated with the increased humoral responses observed in this study. A direct role played by IL-10 in the development of antibody responses in our animal models remains to be confirmed by using IL-10-neutralizing antibody or IL-10–deficient mice.

The use of IL-21 in concert with IL-2 as an adjuvant to T-cell therapy resulted in systemic antitumor immunity. Long-term immunity was shown by the protection of treated hosts against tumor rechallenge, increased numbers of memory T cells, and significantly elevated serum levels of both IFNγ and IL-10. In addition, it is well accepted that serum antibody levels and its persistence are responsible for the establishment of long-term immunity (23–25).

To define the possible mechanisms underlying the antitumor effects of antibodies, we performed a series of experiments using the immune sera collected from the animals subjected to T-cell + IL-2 + IL-21 therapy, and the use of B-cell–deficient mice provided direct evidence that humoral responses truly contribute to T-cell + IL-2 + IL-21–elicited antitumor immunity.

Figure 6. Continued. C, IgG2b-enriched immune sera induce tumor-specific cytotoxicity. Viable MCA205 tumor cells (0.5 × 10⁶) were put in 4 mL test tubes in 450 μL CM plus 50 μL sera from two nontreated control mice, respectively, as indicated or 50 μL sera from three immune mice subjected to T-cell + IL-2 + IL-21 therapy. Cells were incubated on ice for 1 h. After spin, culture supernatant was discarded and 450 μL fresh CM plus 50 μL rabbit complement were added to each tube followed by incubation in a 37°C water bath for another 1 h. Viable cells were then counted under the microscope after trypan blue staining. Percentage (%) of viable cells was calculated by dividing the remaining viable cells with 0.5 × 10⁶. Some groups were left untreated (CM only) or treated with complement (C) alone. Pan-02 tumor cells were used for specificity control. Data are representative of two independent experiments conducted. D, immune serum and complement-mediated cytotoxicity analyzed in 96-well culture plates and determined using the Quick Cell Proliferation Assay kit. This assay was performed according to the manufacturer’s instructions. MCA205 tumor cells were cultured with the sera of six T-cell + IL-2 + IL-21–treated mice, respectively (mice 1 and 2 were used in experiment 1 (Expt.1) and mice 3, 4, 5, and 6 were used in experiment 2 (Expt.2)), followed by culture in complement. Some groups were cultured in CM without sera and complement or with complement alone. Absorbance values were then measured via a multimwell spectrophotometer to evaluate cell lysis.
In lysis, it was reported that antibody-dependent cell cytotoxicity involves NK cells (42) and neutrophils (43). Cell depletion or the use of cell subset–deficient animals would show the roles played by these cells in antibody-mediated tumor cell destruction.

Use of B-cell–deficient mice as recipients in this study strongly involved that host B cells are involved in T-cell + IL-2 + IL-21–induced antitumor immunity because the adoptively transferred TDNL cells were 95% to 99% CD3+ T cells. To eventually test the role that may be played by the tiny residual B-cell population in the infused cells, TDNL cells would need to be generated from B−/− donors or from wt donors depleted of B cells before adoptive transfer. In addition, using congenic animal models and/or carbo-

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Takekazu Iuchi, Seagal Teitz-Tennenbaum, Jianhua Huang, et al.


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